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THE TITRATION OF DIPHTHERIA TOXIN BY THE FLOCCULATION METHOD.

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In the past few months we have examined several thousand samples of toxin and found that the method of Ramon (1922) applied to toxin titration can be carried out by assistants with but little previous training. The method is so simple that it appears advisable to place on record a detailed account of our technique, for as Glenny Pope and Waddington (1925) have shown, the strength of a toxin determined by the flocculation method is of more value to the immunologist than the various animal determinations. In this paper we deal only with the test as applied to the titration of toxin.

In a previous paper Glenny and Okell (1924) suggested a new unit, the Lf value of a toxin, which they defined as the amount of toxin established by flocculation to be equivalent to 1 unit of antitoxin. We would now suggest the following as the most accurate definition:—

> *An Lf dose of toxin is the amount of toxin per unit of a certain chosen antitoxin in the mixture that flocculates more rapidly, under the same conditions, than all other mixtures containing other amounts of the same toxin per unit of the same antitoxin.*

In practice it is more convenient to keep the volume of toxin constant and to determine the number of Lf doses per c.c. of a toxin by finding the number of units of antitoxin per c.c. of toxin which gives the most rapidly flocculating mixture. For general routine purposes, to a series of narrow tubes of uniform bore containing 1·0 c.c. of the toxin to be examined we add a graduated series of quantities of antitoxin. The addition to the series is completed as rapidly as possible. The toxin and antitoxin in each tube are mixed by inverting the tube, the contents being protected from the thumb by means of a piece of non-absorbent paper about an inch square; a separate piece of paper is used for each tube. The tubes are immersed to a uniform depth in a water bath kept at about 50° C., and examined at suitable intervals until a fine precipitate appears in one tube; this precipitate rapidly becomes more pronounced and flocculates; precipitates soon appear in adjacent tubes.

The use of the Trevan microsyringe enables small volumes of antitoxin to be measured accurately. The amount of the liquid delivered by the micro-syringe is controlled by the distance the plunger is made to move; this is accurately gauged by means of a micrometer head. By careful selection, syringes may be chosen that will deliver 0·1 c.c. for a given number of turns. Still further choice can link the syringe with the strength of the standard antitoxin in use. For example, 1 unit of antitoxin containing 280 units per c.c. is delivered by each quarter turn of a micrometer head attached to a syringe delivering 0·1 c.c. for every seven turns. If enough syringes of suitable bore are not available the standard antitoxin is kept diluted down to a suitable strength. Thus we frequently use "8 turn" syringes with serum diluted to contain 200 units per c.c. One complete turn of the micrometer delivers 2·5 units, or 1 unit is delivered by turning the fine adjustment through 20 divisions. The use of the micrometer syringe does not increase the time taken to prepare the tests when several series of tests are made with the same serum and the accuracy of manipulation is greatly increased. By adding very small volumes of undiluted serum the total volume of liquid in each tube does not differ sufficiently to affect the test. If the antitoxin is added in the form of larger volumes of diluted serum the total volume in each tube must be made equal.

Table I. shows the typical method of titrating a toxin of unknown strength. A preliminary test (1) is made at very wide limits; to a series of 5 tubes each containing 1·0 c.c. of toxin we add 2, 5, 10, 20 and 50 units of antitoxin. In the particular example recorded we noticed after the mixtures had been kept at 50° for 25 minutes that flocculation had occurred in the mixture containing 20 units of antitoxin (indicating mixture), but not in the tubes on either side containing 10 and 50 units respectively (limiting values). From this we concluded that the strength of the toxin lay between 10 and 50 Lf units per c.c. The second test consisted of a series of 1 c.c. quantities of toxin to which were added 10, 15, 20, 30, 40 units of antitoxin. In this series the mixture containing 30 units of antitoxin flocculated

TABLE I.

Typical titration of a toxin.

Test	1	2	3	4	5
Scale	E	D	C	B	A
Approximate differences between successive mixtures.	100%	50%	20%	10%	5%
Number of units of AT. per c.c. of toxin. [+ = flocculation, − = no flocculation at the time of examination (Tf).]	50 −	...	50 −	...	...
	...	40 −	40 −	...	...
	...	...	35 −	...	...
	...	30 +	30 −	30 −	...
	...	...	...	28 −	...
	...	...	...	26 −	26 −
	...	...	25 +	...	25 −
	...	...	...	24 +	24 −
	...	...	...	...	23 +
	...	...	...	22 +	22 −
	...	...	...	...	21 −
	20 +	20 −	20 −	20 −	20 −
	...	15 −	...	...	...
	10 −	10 −	...	...	...
Tf at 50° C. (in minutes)	25	24	20	18	15
Indicated value (in Lf units per c.c.)	20	30	25	22-24	23
Limiting values	10-50	20-40	20-30	20-26	22-24

before any other; 30 units therefore was the indicated value with 20 and 40 as the limiting values. Subsequent tests reduced the extent of the limiting values and increased the accuracy of the test until a value of 23 Lf units per c.c. was recorded as correct to 5 per cent. It will be seen from the table that without any discrepant tests the indicated values for the same toxin have been given as 20, 30, 25, 22-24 and 23. It is essential therefore when recording preliminary tests to quote the scales upon which the tests have been made. We have found it helpful to make use of a series of letters, and unless the strength of the toxin has been determined to narrow limits we always qualify the strength quoted by a scale letter (see table I.). The successive values mentioned above are quoted by us as 20 E, 30 D, 25 C, 22-24 B and 23 A.

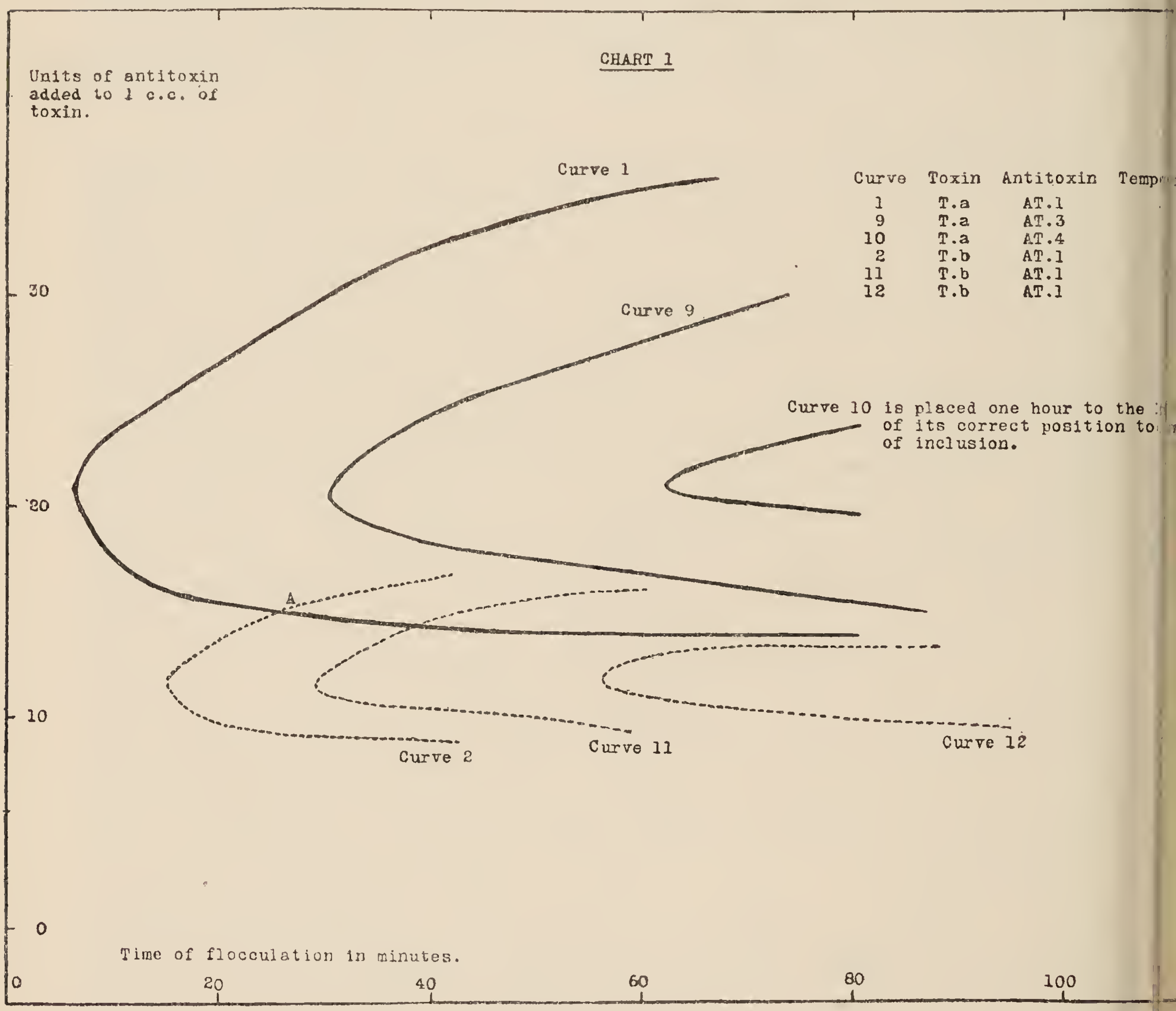
In practice it is not always necessary to make so many tests before reaching the A scale. We have not found an unconcentrated toxin stronger than 30 Lf units per c.c.; we have consequently recently adopted for the preliminary testing of unknown toxins a scale of 2, 5, 10, 20, 30 and have included also a mixture containing 50 units for the detection of non-specific flocculation (see below). Again, it is possible by noting which of the limiting tubes flocculates before the other to decide upon which side of the indicating tube the correct value lies. Referring again to table I., in the second series the tube containing 30 units flocculated in 24 minutes; one minute later the tube containing 20 units flocculated, but the 40-unit tube did not flocculate until some time later. This result indicated that the limiting values could be taken to be 20-30 units and the third test on the C scale was unnecessary. More frequently, when toxin prepared in a routine way is being tested, close limiting values are known; such toxin would be tested in a preliminary series for 10, 15, 20, 25 units, and the second test would often be transferred to the B or even A scale.

When a number of tests are being made, the actual time taken to prepare the mixtures of toxin and standard antitoxin for one series of tubes is not greater than five minutes. The figures given in table I. for Tf (a convenient symbol for flocculating time) show the rapidity with which tests can be made. It is often possible to make a first rough approximation of the strength of a toxin within 30 minutes of commencing the test and accurate values are known within from 1 to 2 hours. A single worker can prepare and read 50 series of tests in one day.

Time of flocculation.

The rate at which a mixture of toxin and antitoxin flocculates is the essential feature of the Ramon test. Flocculation will occur when toxin and antitoxin are mixed in different proportions, but under any given conditions the rate of flocculation depends upon the relative proportion of toxin and antitoxin and a "balanced mixture" wil precipitate before all others. The term "neutral mixture" is frequentl used when implying neutrality (*i.e.* absence of toxicity) to animals for the sake of clearness therefore we use the expression "balance mixture." An "indicating mixture" is not necessarily a balance mixture, but is the most rapidly flocculating mixture of the serie prepared.

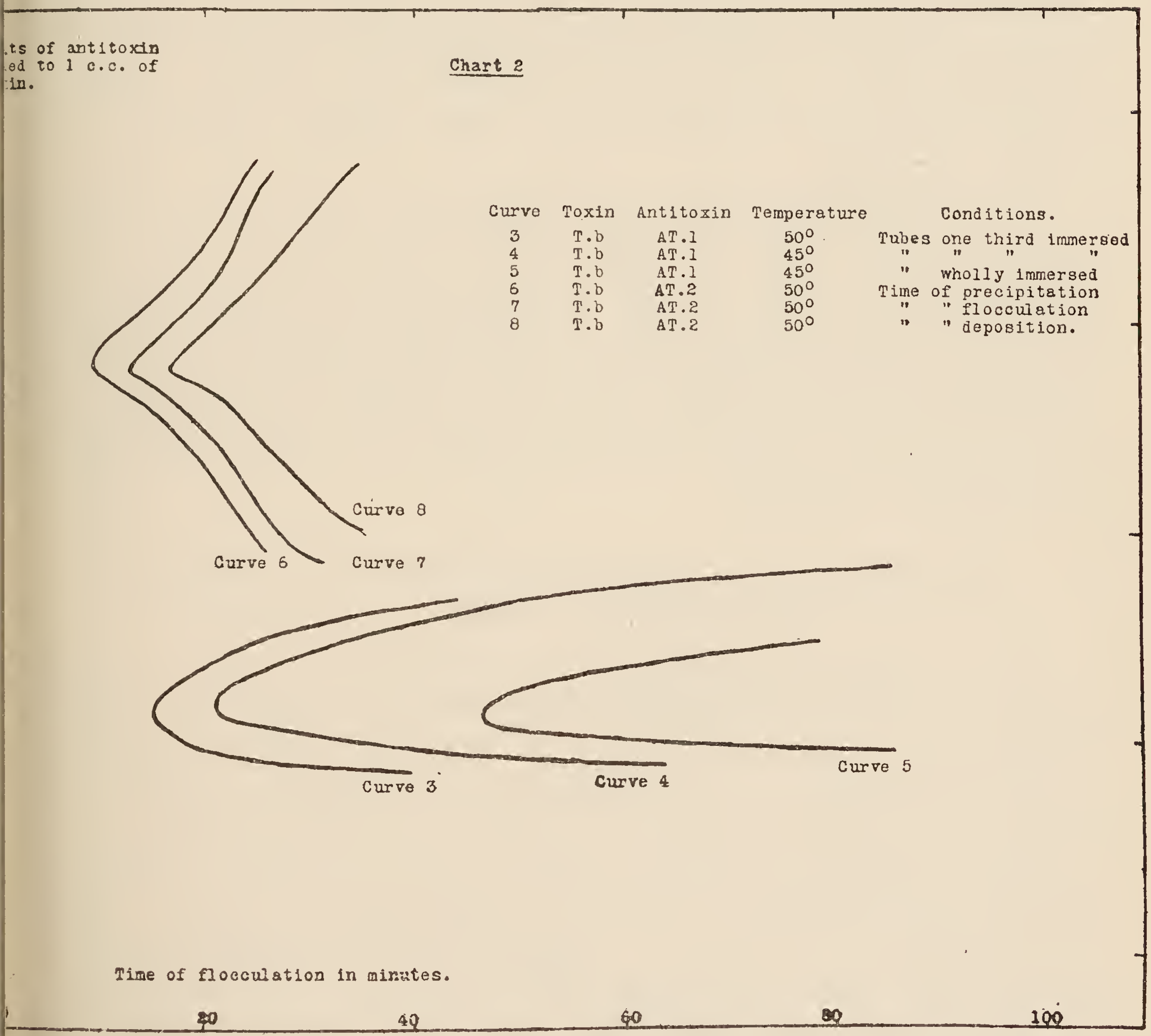
The relationship between time of flocculation and composition c flocculating mixtures, may be shown graphically as on chart



Curve 1 shows the time of flocculation, under certain conditions temperature, etc., of mixtures containing 1 c.c. of toxin T.a. and varyi amounts of antitoxin AT.1. It will be seen that flocculation occurr within one hour in all mixtures containing between 14 and 35 un of antitoxin to each c.c. of toxin. The mixture containing 21 un of antitoxin per c.c. of toxin flocculated in a shorter time than others. The toxin therefore contained 21 Lf doses per c.c. Curve shows the time of flocculation for mixtures of a second toxin T.b. wi the same antitoxin. The point (A) at which the curves for the t different toxins cross shows that a mixture of 1 c.c. of toxin T.a. wi 15 units of antitoxin AT.1. will flocculate in the same time (minutes) as a mixture of 1 c.c. of toxin T.b. and the same amou

(15 units) of the same antitoxin (AT.1.), although toxin T.a. is obviously almost twice as strong as toxin T.b. This fact shows that the time in which a single mixture of toxin and antitoxin flocculates cannot be taken as any indication of the strength of a toxin, but that it is always necessary to test a series of mixtures to determine the composition of the balanced mixture, *i.e.* the indicating mixture in an infinite series.

Rate of flocculation depends mainly upon the temperature at which mixtures are kept and the depth of water surrounding the tubes. Curves 3, 4, and 5 on chart 2 are flocculation curves for one toxin T.b.



Curve	Toxin	Antitoxin	Temperature	Conditions.
3	T.b	AT.1	50°	Tubes one third immersed
4	T.b	AT.1	45°	" " " "
5	T.b	AT.1	45°	" wholly immersed
6	T.b	AT.2	50°	Time of precipitation
7	T.b	AT.2	50°	" " flocculation
8	T.b	AT.2	50°	" " deposition.

and one antitoxin AT.1.; curve 3 represents a temperature of 50° C. and curve 4 of 45° C. Curve 5 has also been produced at a temperature of 45° C. but this curve represents a series in which the tubes were "fully immersed," *i.e.* to the top of the column of fluid in the tube, while those represented by curve 4 were immersed only to a third of their depth. The marked difference in flocculation time between curves 4 and 5 shows the great effect produced by agitation caused by convection currents. In any series of mixtures at narrow limits discrepant readings always occur unless considerable care be taken to ensure uniformity of conditions in all tubes of the series. Equal volumes of the same mixture in tubes of different width, 0·5 cm.

and 0·75 cm. in diameter, flocculated in 40 minutes and 47 minutes respectively under identical conditions when one-third immersed, and in 54 minutes and 74 minutes respectively when fully immersed. 2·0 c.c. and 1·0 c.c. volumes of the same mixture in tubes of the same diameter flocculated in 42 minutes and 47 minutes side by side in the bath, the tubes being equally, and the columns of liquid unequally, immersed. If, however, uniformity of heating and of convection currents is ensured by totally immersing the tubes, the volume of mixture, provided there is sufficient to give a visible precipitate, does not appear to influence the rate of flocculation; 5·0 c.c. and 1·0 c.c. volumes of the same mixture, completely immersed, both flocculated in 40 minutes; it is, however, difficult to ensure a simultaneous rise of temperature in very unequal volumes of liquid in the same bath.

It is essential to have all the tubes in one series uniform since mixtures in wide tubes flocculate more slowly than in narrow, and to have exactly the same volume of liquid in each tube since the depth of immersion affects the rate of flocculation. The thickness of the bottom of drawn out glass tubes varies considerably, so that unless the tubes are carefully selected the amount of liquid immersed will not be equal in tubes immersed to the same depth, even though the diameter of the tubes is the same.

Relation between time of flocculation and strength of fresh toxins.

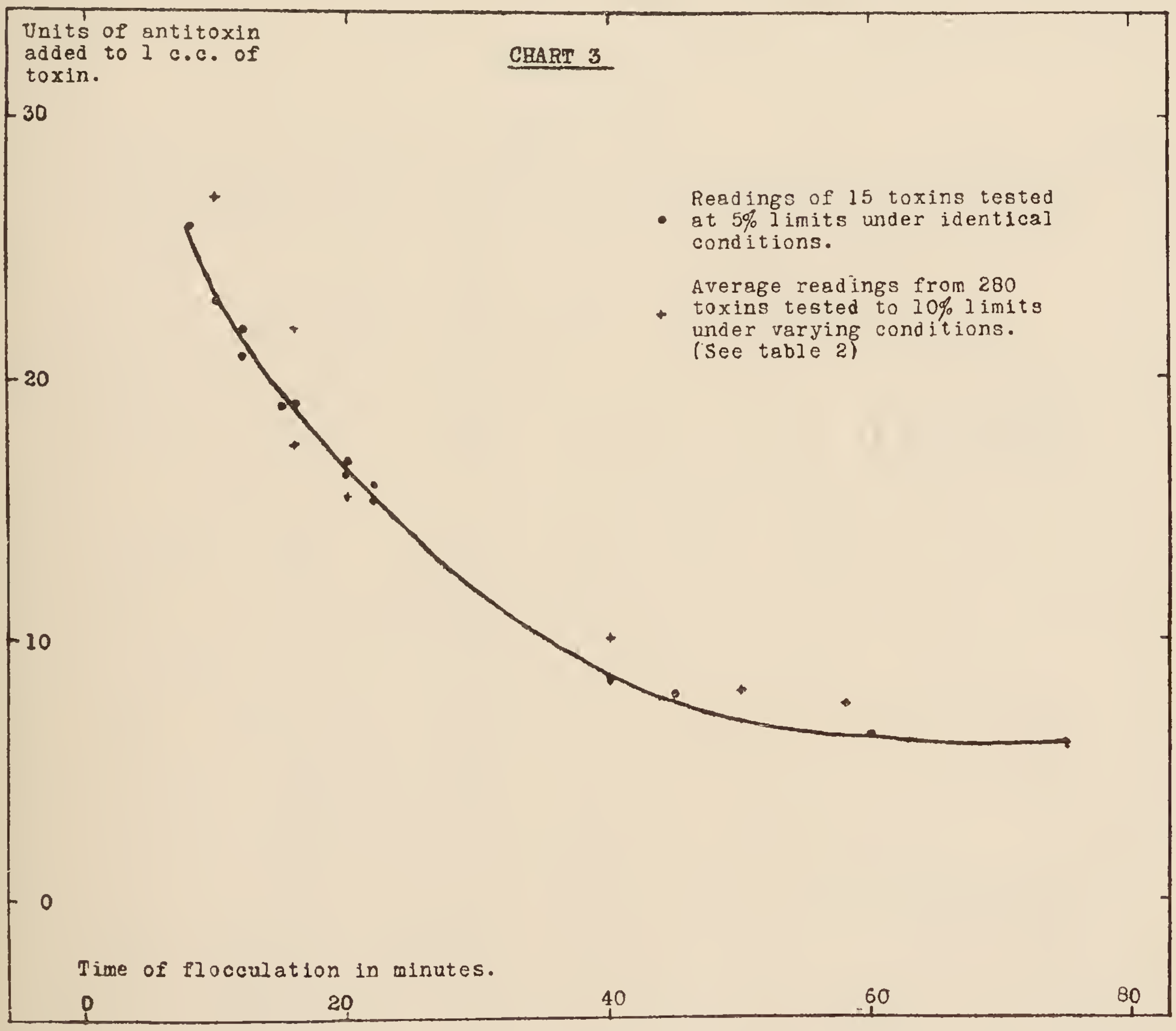
The number of conditions upon which the rate of flocculation depends is so great that Tf., the flocculation time for a balanced mixture, has only a relative value. The relative rates of flocculation of different toxins can be compared if the toxins are titrated under identical conditions. A group of toxins, all prepared during a period of three months from the same strain but on a variety of media were tested simultaneously on scale A, *i.e.* the antitoxic content of adjacent tubes differed by 5 per cent. The Lf and Tf. are plotted on chart 3 which shows, since the points all lie on a smooth curve, that the rate of flocculation of fresh toxins is a function of the strength of the toxin. The strongest toxin flocculates most rapidly, *e.g.* that containing 26 Lf units per c.c. flocculated in 8 minutes, while the weakest toxin in this series containing 6 Lf units per c.c. did not flocculate until 75 minutes had elapsed. With such varying conditions as arise from day to day with routine tests the connection between Tf. and strength is not so uniform. To evaluate any batch of toxin it is necessary for conditions to be constant in one series of mixtures but it is not necessary for them to be constant between different series. In practice a test upon one toxin may have been made at a temperature several degrees lower than that at which another toxin had been tested. The temperature of the air above the bath differs markedly on different days, and the diameter of all tubes in one series of tests may be different from the diameter of all tubes used in another series. Table II.

TABLE II.

Showing the number of batches of toxins of various strengths flocculating within a given time when tested against standard antitoxin AT.1.

Lf units per c.c.	Under 15 mins.	20 to 25.	30 to 35.	40 to 50.	1 to 1½ hour.	2 hours or more.	Average time.	Calculated times from curve on chart 3.
4 to 6	...	...	...	...	3	3	100	...
6 ,, 8	...	...	...	5	17	4	75	75
8 ,, 10	...	1	5	22	7	...	50	45
10 ,, 12	...	...	12	18	...	...	40	37
12 ,, 14	...	15	21	23	...	...	30	30
14 ,, 16	5	20	10	3	...	...	25	26
16 ,, 18	9	18	2	2	...	...	20	22
18 ,, 22	21	14	...	...	...	...	15	16
22 ,, 28	14	6	...	...	...	...	15	12
28	4	...	...	...	...	...	10	7

and the additional points marked upon chart 3 show that even under such variable conditions of test there is still a definite relation between time of flocculation and strength of toxin. The time of flocculation of a toxin depends also upon the antitoxin used for titration. Thus in chart 1, curve 1 shows that Tf. of toxin T.a. titrated against antitoxin AT.1. was 6 minutes, and curve 9 shows that under similar conditions Tf. for the same toxin against a different antitoxin AT.3., was 30 minutes.



Standard antitoxic serum.

Curves 6, 7 and 8 in chart 2 show the time of precipitation, coarse flocculation, and deposition respectively in mixtures of the same toxin T.b. as in curve 3 and another antitoxin AT.2. kept at a temperature of 50° C. Thus it is seen that when the same toxin (T.b.) is titrated against antitoxin (AT.1.) a balanced mixture is produced by the addition of 11½ units of antitoxin to 1 c.c. of toxin; if another antitoxin (AT.2.) is used, 28 units of antitoxin are needed to form the balanced mixture. The estimation of strength of this toxin depends therefore upon the antitoxin against which it is titrated. It is necessary therefore to refer in our definition of an Lf dose to a "certain chosen antitoxin." We have chosen 1 Ehrlich unit of a certain antitoxin (AT.S.) determined by animal methods as the basis of standardisation; if now in place of titrating toxin T.b. against 1 Ehrlich unit of antitoxin AT.2., we titrate AT.2. against 1 Lf unit of toxin T.b. we obtain an *in vitro unit* value for AT.2. If the *in vitro unit* value is a definite strength then it should be possible to titrate other toxins by the flocculation method equally against 1 *in vitro unit* of AT.S. (accepted as equal to 1 *in vivo unit*) as against 1 *in vitro unit* of AT.2. independently of its *in vivo value*.

TABLE III.

Showing the strength of a number of different toxins in Lf units per c.c. determined by titration against in vitro *units of different sera.*

Antitoxin used.	AT.S.	AT.2.	AT.7.
In vivo value	280	260	340
In vitro value	280	110	260
Ehrlich units per *in vitro* units	1·0	2·36	1·30
Lf units per c.c.	9·0	8·2	9·2
	3·2	...	3·2
	6·4	...	7·4
	5·6	5·5	...
	6·7	6·7	...
	5·0	5·0	...
	9·8	9·9	...
	11·2	10·4	...
	8·4	8·2	...
	5·6	5·5	...
	7·0	6·6	...
	4·8	4·7	...
	7·7	7·7	...
	5·6	6·0	...
	3·6	3·8	...
	5·6	5·5	...
	17·5	16·9	16·3
	8·25	7·0	8·6
	27·0	26·2	27·0
	8·25	7·8	9·3
	21·0	18·6	20·8
	16·0	16·8	16·7

The *in vitro* and *in vivo* value of AT.S. is 280 units per c.c., the *in vivo* value of AT.2. is 260 units and the *in vitro* value 110 units per c.c. Table III. shows a series of titrations of 22 different toxins; these toxins varied in strength from 3·2 to 27·0 Lf units per c.c. and included toxins produced from tryptic digest of horse muscle, peptic digest of veal and infusion of bullock heart muscle with various peptones added. There is close agreement between the Lf values of each of these 22 toxins titrated against 1 Ehrlich unit of AT.S. and that titrated against 2·36 Ehrlich units of AT.2. A few determinations against a third antitoxin AT.7. are also included in the table.

When choosing a standard serum it is necessary therefore to standardise the antitoxic value of the serum by flocculation methods and to determine the *in vitro* value of the serum as distinct from the *in vivo* value. We would here point out that we think it advisable at present to use only such provisional terms as "in vitro unit," because we do not yet know the full significance of the differences between *in vivo* and *in vitro* values of sera or of non-specific flocculation, or of double zones.

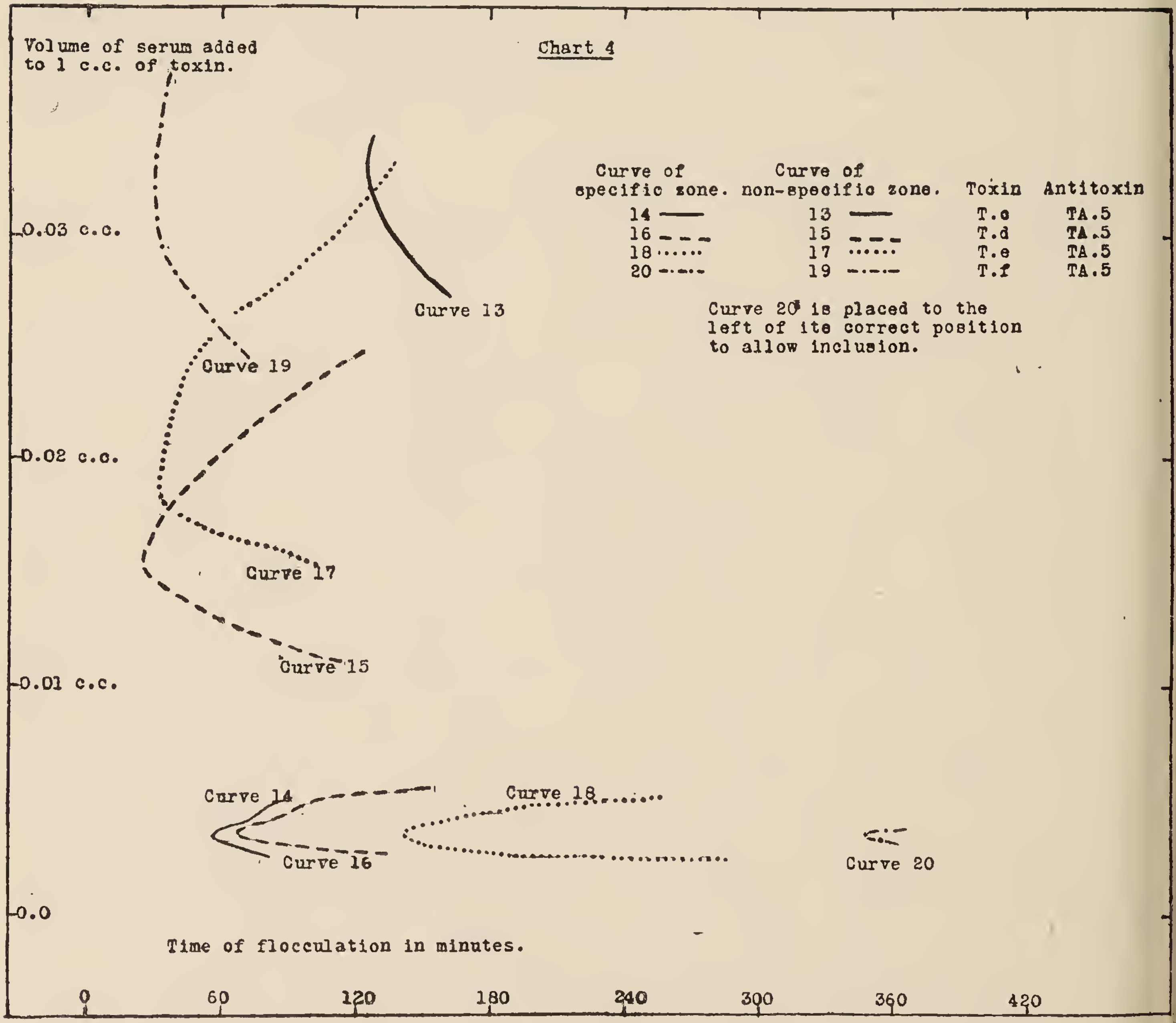
In choosing a standard serum there are several other important points to be observed, *e.g.* rate and intensity of flocculation, suitable strength and freedom from such complicating factors as double zones. For rapid working we adopt the technique described in this paper exposing our mixtures to a temperature of 50° C. and making use of a standard serum that flocculates with extreme rapidity. For more accurate work there are two definite courses open: (1) accept the average result of many tests rapidly made, (2) delay the rate of flocculation by lowering temperatures, dilution, reducing convection or by choice of standard serum. It is possible that subsequent work will show that there is a third course: choice of a standard serum that gives a very narrow zone of flocculation. Intensity of flocculation is of importance because certain sera when fresh and all old sera examined yield only a poor precipitation difficult to observe. It is our experience that rapidly flocculating sera give larger precipitates. It is of advantage to use standard sera of picked strength; it will be noticed that the values of the sera used for the titrations recorded in table II. are all very low. If we made use of stronger sera, *e.g.* between 1000 and 2000 units per c.c., the amount to be added to each c.c. of toxin would be so small as to necessitate dilution. Such sera would be of use only when titrations are being made of highly concentrated toxins. There is an obvious advantage in holding different batches of standard sera of such strengths that the titration of any sample of toxin met with will involve convenient measurements.

Certain sera are found to possess *two zones of flocculation*. By titrating against several different toxins it is possible to determine that one zone is specific and the other non-specific. Thus a certain batch of serum AT.4. when titrated against toxin T.c. appeared to contain either 30 or 290 *in vitro* units per c.c. according to which zone was accepted. Another toxin T.d. gave values of 65 and 290 for this serum, a third toxin T.e. gave 55 and 290, and a fourth T.f. 30 and 290. The flocculating times for the indicating tubes of both zones are given in table IV. and the relation between amount of serum present and times of flocculation are traced graphically on chart 4.

TABLE IV.

Showing the composition and the flocculating times of mixtures of serum AT.5. with four different toxins.

	Amount of serum per 1 Lf unit of toxin.			
Toxin.	T.c.	T.d.	T.e.	T.f.
Lf units per c.c.	22	15	9·25	5·5
Specific zone	0·00345 c.c.	0·00345 c.c.	0·00345 c.c.	0·00345 c.c.
Non-specific zone	0·0333 c.c.	0·0154 c.c.	0·0182 c.c.	0·0333 c.c.
Flocculating times (in minutes) of indicating mixtures.				
Specific zone	54	69	143	Several days
Non-specific zone	125	25	32	40



Curves 14, 16, 18 and 20 show that a balanced mixture is produced by mixing 0·00345 c.c. of serum AT.4. with 1 Lf dose of any of the 4 toxins, while curve 13, 15, 17 and 19 have no common indicating mixture. It will be seen that non-specific flocculation occurred in 32 minutes with toxin T.e.; specific flocculation did not appear until nearly two hours later. Misleading results would thus follow the use of this serum.

It is important therefore to take every care to ensure that a standard serum is free from non-specific flocculation. When choosing a new standard it is not sufficient to make a series of careful titrations against a single toxin to determine the *in vitro* value of the serum; a second toxin should be used to make certain that the point of earliest flocculation observed represents specific flocculation of diphtheria toxin and antitoxin. In addition it is advisable to explore the new serum over a large range of doses. If a second point of flocculation is observed the serum should not be used.

Accuracy of test.

The method that we have described has been devised to produce rapid results and to enable large numbers of tests to be done. The degree of accuracy of the rapid method is considerable. Table V. shows that when 65 batches of toxin were tested at wide limits (20 per cent.), 61 gave the same indicating tube when the test was repeated and 4 gave adjacent tubes. At narrower limits of 5 and 10 per cent., 144 out of 179 showed the same indicating tube in the repeat test and 160 out of 179 were correct to within 10 per cent.

Table V.

Showing the degree of conformity between two tests upon each of 244 batches of toxin.

Scale of test and limits to which tests were made.	Number of batches of toxin of which the repeat test gave—			
	The same value.	5 per cent. difference.	10 per cent. difference.	20 per cent. difference.
C=20 per cent.	61	...	...	4
B=10 ,,	96	...	17	...
A=5 ,,	48	16	2	...

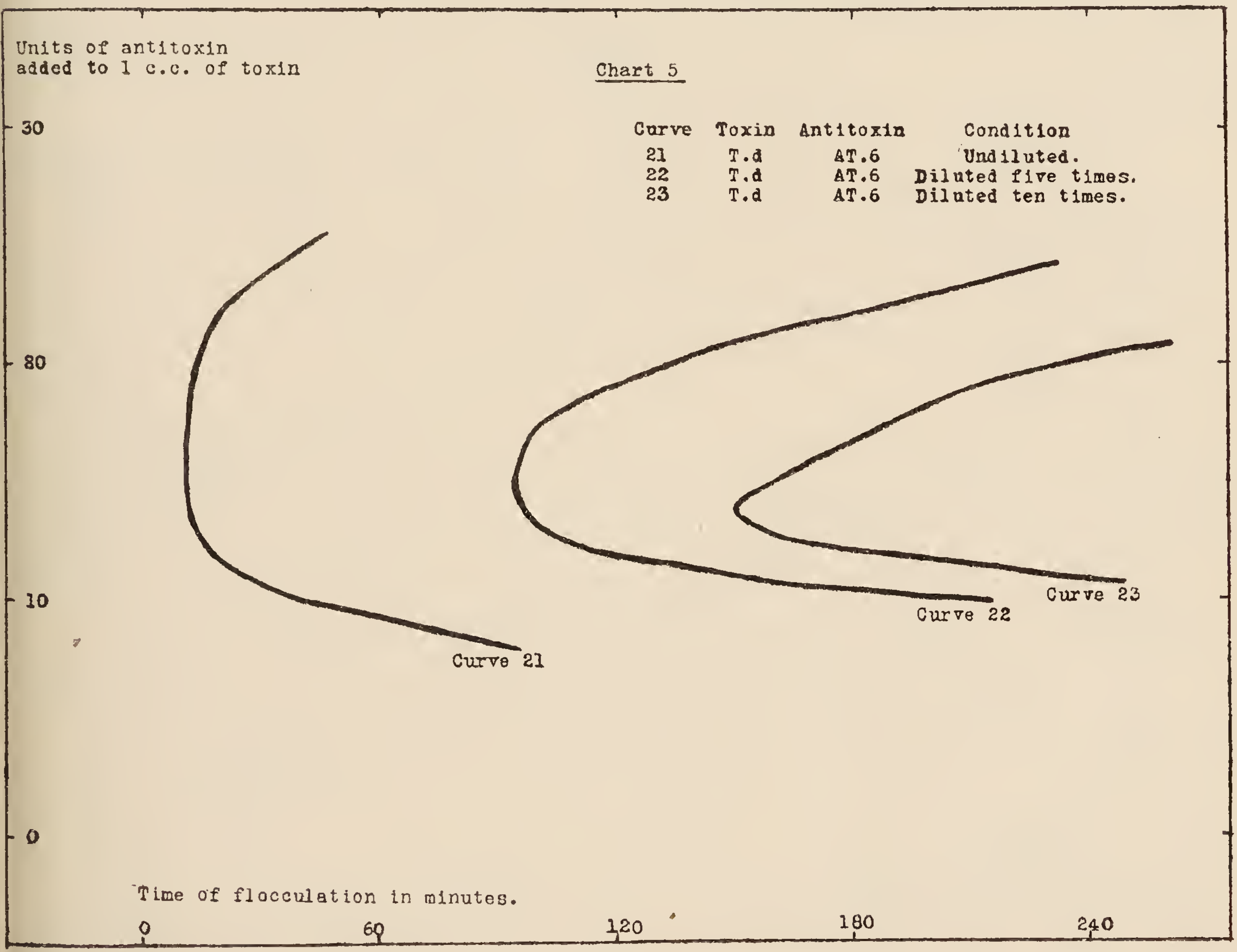
The ease and rapidity with which toxin can be tested with an accuracy of 10 per cent. enables frequent retests to be made upon bottles of toxin during use. It is our custom to blend together batches of toxin containing between 20 and 100 litres and these blended batches are filtered into 4-litre containers. It is possible that one bottle may differ from another owing to some differences in filter candles used or other obscure causes, and different bottles may deteriorate at different rates. In order to detect such differences we test each bottle after filtration and immediately before use for immunisation, and the last remains of toxin in each bottle are tested to detect possible deterioration during use. In a series of 127 bottles representing 14 blended batches of toxin only 4 gave a value more than 10 per cent. different from the average value for each batch.

When accurate results are needed flocculation must be delayed. Curves 1, 9 and 10 on chart 1 show the relation between the time of flocculation and composition of mixtures containing one toxin (T.a.) and three different antitoxins (AT.1., AT.3. and AT.4.). The minimum flocculating times are 6, 30 and 122 minutes respectively. The accuracy to which a toxin can be titrated depends mainly upon two conditions (i) the ease with which the first mixture to flocculate can be chosen from adjacent mixtures, (ii) the extent to which uncontrolled conditions may so accelerate or retard flocculation in one tube or another that the flocculating time of the balanced mixture is delayed beyond that of other mixtures. Uncontrolled conditions such as unequal rates of heating in different tubes may delay flocculation for several minutes; it is obvious that such a delay is of less significance in a series of slowly flocculating mixtures than in a series of fast ones. For the purposes of comparison we have chosen as an index of possible accuracy the extreme range of antitoxic content of all mixtures flocculating within 5 minutes of the first tube to flocculate. Thus from curve 1 it is seen that all mixtures containing between 16·6 and 24·0 units of antitoxin AT.1. per c.c. of toxin T.a. flocculate within 6 to 11 minutes of mixing. When another serum AT.3. is used the first mixture flocculates in 30 minutes and within the next 5 minutes all mixtures containing between 18·7 and 23·0 units per c.c. of toxin have flocculated. Closer limits are obtained with a still slower serum AT.4.; the first mixture flocculates in 122 minutes and only those mixtures containing between 20·5 and 22·0 units of antitoxin flocculate within the next 5 minutes. Thus it is seen that more accurate but slower results can be obtained by using such a serum as AT.4. in preference to AT.1. It must be pointed out, however, that the rapidity with which flocculation occurs in mixtures other than the balanced mixture is not simply a function of the minimum time o flocculation. With certain sera flocculation may occur almos simultaneously in a number of tubes.

The increased accuracy of testing when mixtures are kept at lowe temperatures is shown graphically on curves 2, 11 and 12 on chart showing the relation between time of flocculation and composition o mixtures of another toxin T.b. with antitoxin AT.1. when the mixture are kept at 50°, 35° and 25° C. The times of flocculation at the differen temperatures were 15, 29 and 56 minutes, and the antitoxic content o mixtures flocculating within 5 minutes were 9·7 to 13·6, 10·7 to 13·

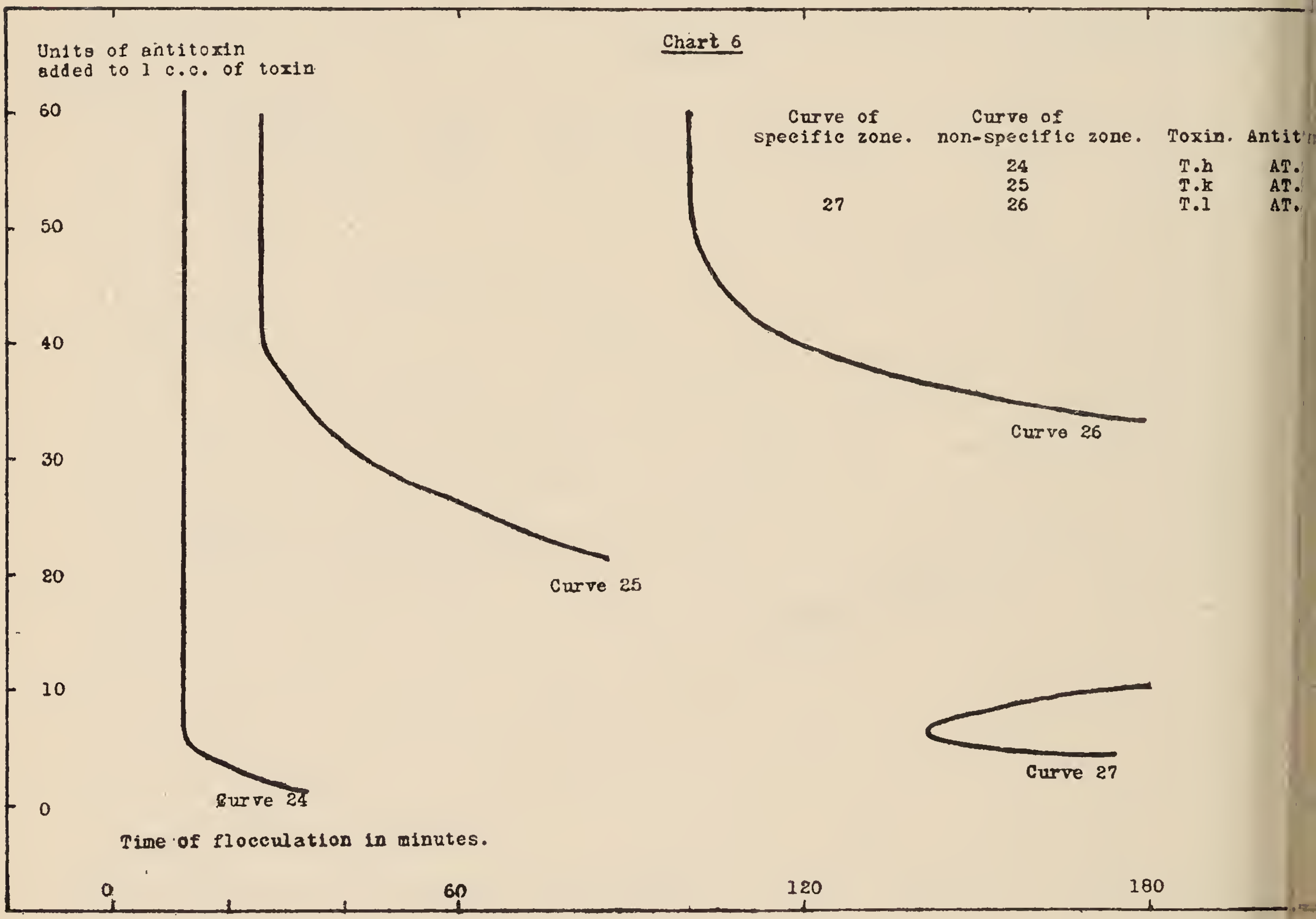
10·7 to 12·7 units per c.c. of toxin Delaying flocculation by reducing the temperature of incubation from 50° to 25° C. has halved the extent of "spread" of flocculation. It has already been shown on curves 4 and 5 of chart 2 that a similar end may be gained by totally immersing the tubes.

Flocculation can also be delayed by diluting the mixture of toxin and antitoxin with saline. A serum AT.6. was chosen with so rapid a "spread" of flocculation that all mixtures containing between 13·5 and 19·0 units per c.c. of a toxin T.d. appeared to flocculate simultaneously when incubated at 50° C. By diluting the mixture five- and ten-fold, flocculation was delayed from 12 minutes to 94 and 150 minutes, and the "spread" occurring within 5 minutes was reduced from between 11·7 to 22·0 units to between 13·2 and 17·2 and 13·0 to 14·6 units (see curves 21, 22 and 23 on chart 5). In these experiments the same series of amounts of serum were added to 1 c.c. of undiluted toxin, to 5 c.c. of a five-fold dilution and to 10 c.c. of a ten-fold dilution, but only 1 c.c. of each mixture was incubated.



Non-specific flocculation.

We have already shown (chart 4) that certain sera may exhibit two zones of flocculation when mixed with any toxin. With such sera it is not always possible to judge which zone is specific unless titrations are made against other toxins. Toxins have also been found which show non-specific flocculation. With certain of these toxins it is a simple matter to determine that the flocculation is non-specific because it takes place most rapidly in mixtures containing a large excess of antitoxin. We have occasionally been able to correlate the appearance of non-specific flocculation with slight bacterial contamination during the preparation of the toxin. Curves 24, 25 and 26 on chart 6 show typical examples of the connection between the time of non-specific flocculation and the composition of mixture. It will be seen that mixtures containing 50 or more units of antitoxin to each c.c. of toxin flocculate as rapidly as, or more rapidly than, mixtures containing less antitoxin. It will be also seen that the minimum time of flocculation bears no such relation to the apparent strength of the toxin as that shown on chart 3.



Some toxins showing non-specific flocculation may also exhibit a specific zone of flocculation. An example of this is seen on chart 6 where curve 26 shows the non-specific and curve 27 the specific flocculation of toxin T.l. against antitoxin AT.1. Toxins T.h. and T.k. showing only a non-specific, and no specific flocculation (curves 24 and 25) were titrated by blend with a known toxin, and were found to contain less than 2 Lf units per c.c. We have also occasionally seen a second flocculation appear in a mixture in which the first (non-specific) flocculation has already deposited.

Several toxins after concentration with acetic acid have shown two limited zones of flocculation. With these concentrated toxins there has been no rapid spread of flocculation to mixtures containing excess of serum to indicate non-specific flocculation. Titrations against other antitoxins show one constant and one varying zone; blending with other toxins of known strength will also indicate which is the specific zone. This method of blending is always resorted to when there is any doubt about the titration of a toxin.

SUMMARY.

1. The technique of the Ramon test is described by which it is often possible to make a first rough approximation of the strength of a toxin within 30 minutes of commencing the test, and accurate values are known within from 1 to 2 hours. A single worker can with ease prepare and read 50 series of tests in one day.

2. Ninety per cent. of tests made in this manner are correct to within 10 per cent.

3. The Lf value of a toxin is now defined as the amount of toxin per unit of a certain chosen antitoxin in the mixture that flocculates more rapidly under the same conditions than all other mixtures containing other amounts of the same toxin per unit of the same antitoxin.

4. The antitoxins against which the toxins are titrated must be standardised by flocculation methods. One Lf unit of toxin is the equivalent of 1 Ehrlich unit of one antitoxin or as much as 2·5 Ehrlich units of another antitoxin.

5. Some batches of antitoxin show a second zone of flocculation (non-specific) and should be avoided for standard purposes.

6. Standard sera should be chosen for use according to the type of tests to be made. A slowly flocculating serum should be used for accurate work.

7. The time of flocculation of mixtures can be delayed and the accuracy of titration increased by dilution or by lowering the temperature of incubation.

8. Certain batches of toxin show non-specific flocculation with excess of antitoxin.

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